THE ASYMMETRY IN TIMING OF DNA REPLICATION INITIATION IN THE MALE *DROSOPHILA* GERMLINE

by

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A thesis submitted to Johns Hopkins University in conformity with the requirements for the degree of Master of Science.

Baltimore, Maryland

April 2019

Abstract

Many adult stem cells undergo asymmetric cell division (ACD) to produce both a self-renewing stem cell and a differentiating daughter cell. Stem cell ACD is crucial for maintaining tissue homeostasis and loss of asymmetric division is associated with both degenerative aging related diseases and cancers. A model system for studying stem cell ACD is the male *Drosophila* Germline Stem Cell (GSC). One key intrinsic factor underlying this asymmetric division in GSCs is the asymmetric segregation of canonical (old) and newly synthesized (new) histones: old histones are retained by the GSC and new histones are selectively segregated to the differentiating Gonialblast (GB) cell. While in theory asymmetric histone segregation could regulate the differential acquisition of new cell fates or processes, the precise mechanisms of how asymmetric histone inheritance regulates ACD is not understood. Here we show that during mitosis the old and new histone enriched sister chromatids show dramatically different properties as the new histone enriched sister chromatids fail to fully condense during mitosis and decondense more rapidly in telophase. The difference in mitotic condensation of sister chromatids is upstream of differences in timing of entry into the next cell cycle, where the GB proceeds into DNA replication before the GSC does. Under conditions that randomize histone inheritance, DNA replication initiation timing is randomized between the daughter cells. Furthermore, conditions that eliminate differential condensation/compaction of sister chromatids abolish DNA replication entry asymmetries. Together, these studies provide evidence that asymmetric histone inheritance induced differential mitotic chromatin compaction plays a role in DNA replication initiation timing, demonstrating a clear downstream effect of asymmetric histone inheritance.

Advisor: Xin Chen, Ph.D.

Preface

The experiments, observations, and conclusions provided herein were products of my project on DNA replication initiation that will fully culminate in May 2019. This research was done at Johns Hopkins University in Baltimore, Maryland in the Department of Biology under the supervision of Dr. Xin Chen. Upon reading fascinating papers produced by this lab on the epigenetic roles of asymmetric stem cell division, I enthusiastically joined this lab to further understand the implications of asymmetric cell division, particularly that of DNA replication initiation.

Biggest thanks to Dr. Chen for introducing me to the fascinating field of epigenetics and developmental biology, funding for all of my experiments, and the many conversations piquing curiosity and critical thinking of ideas in this field. I would also like to thank the Chen lab family for their unwavering support, especially Jonathan Snedeker for his great mentorship and patience, Emily Zion for always providing comfort in times of stress along with our exciting discussions of data, Chinnu Chandresekhara for teaching me the ins and outs of image analysis and quantification, and Matthew Wooten for all the excitement, positivity, and enthusiasm he provided every time we spoke about my data. Furthermore, I want to thank Dr. Kathryn Tifft Oshinnaiye for her tireless academic, emotional, and mental support through all the ups and downs of my project: thank you for always having your office open to me.

Finally, I want to thank my fellow Masters students Alina Corona, Sophia Wu, Vanessa Gonzalez, Zach Zhao, and Akshay Bhamidipati. I can not imagine going through this process with any other group of classmates: you guys are wonderful!

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Chapter 1: To Differentiate or Not To Differentiate

Homeostasis is the key to maintaining health and longevity in all organisms. One of the key processes required for maintaining tissue homeostasis is the regulation of stem cell proliferation and differentiation via asymmetric cell division. Asymmetric stem cell division is the process wherein one stem cell divides into two daughter cells with distinct fates: a self-renewed stem cell and a differentiated cell. Over-proliferation of stem cells can lead to tumorigenesis and cancer[1,2] while under-proliferation of stem cells can lead to tissue degeneration due to the depletion of self-renewing stem cells and their progenitors, which can replace senescent cells[3] (Figure 1A).

Extrinsic and Intrinsic Means of Stem Cell Maintenance

A combination of extrinsic and intrinsic mechanisms underpins asymmetric cell division. Extrinsic mechanisms refer to any external non-cell autonomous factor that influences cellular fate, usually altering cell fate after division. Such extrinsic factors often relate to signaling molecules and systemic factors, particularly signals coming from nearby cells. Intrinsic mechanisms refer to the asymmetric segregation of cellular components that may influence destiny within the daughter cells during mitosis.

Due to the ease that the male *Drosophila* germline provides in visualizing the stem cells and their relation to their environment, this organism provides an excellent model for studying mechanisms behind stem cell maintenance and differentiation of progenitors. It was discovered nearly two decades ago that GSC renewal in the male *Drosophila* germline requires the JAK-STAT signaling pathway[4,5]. The Janus kinase/signal transducers and activators of transcription (JAK-STAT) pathway is the key mechanism for signaling of various cytokines and growth factors[8,9]. When the JAKs are activated via binding of specific ligands, the kinases can then

activate STAT proteins, often through phosphorylation[6,7]. In the male *Drosophila* germline, the JAK is Hopscotch (Hop) and the STAT is Stat92E, both of which are autonomously necessary for GSC renewal as evidenced by the loss of early germ cells in mutants lacking either Hop or Stat92E[4]. The activating ligand of the JAK-STAT pathway is Unpaired (*upd*), an extracellularly secreted protein that was found to be sufficient to increase the GSC population when ectopically expressed[5]. Signaling pathways, such as the JAK-STAT pathway, are often evolutionarily conserved through species, showing that specificity in controlling various extrinsic cell fate decisions is often achieved by molecular concentrations, combinations, and homologs[7] to extrinsically regulate asymmetric cell division. Interestingly, extrinsic mechanisms are not limited to molecular pathways; studies have been shown that environmental factors such as diet along with tissue-extrinsic signals[10] also effects stem cell activity. In the female *Drosophila* germline, neural-derived *Drosophila* insulin-like peptides (DILPs), proteins essential for ovarian response to nutrition, have a direct role in proliferation of germline stem cells[10]. Furthermore, protein-poor diet and resulting decreased insulin signaling conditions negatively affect rates of division and development[10]. Thus, there exist multiple external factors at work that influence cell fate in the germline by reinforcing stemness or inducing differentiation.

In contrast to extrinsic mechanisms, intrinsic mechanisms utilize cell autonomous asymmetric segregation of cellular components that may influence destiny within the daughter cells. Cell intrinsic factors are factors that are present within the stem cell to influence cell fate. These factors can include cytoskeletal components[11,13], translational regulation[11], and protein localization[16]. One of the most studied intrinsic factors regulating stem cell renewal relates to the specific orientation of mitotic spindles within dividing male GSCs. Male GSCs strictly maintain a perpendicular orientation toward the tip of the testis, a property that is necessary for balanced

stem cell renewal[11]. The mechanism behind this phenomenon is maintained by the specific localization of one of the centrosomes to the tip of the testis after centrosome duplication. Null mutants for a centrosome protein that is vital for proper astral microtubule function[12], *centrosomin* (*cnn*), displayed high frequency of spindle orientation defects and an increase in GSC number[11]. There exists asymmetric centrosome inheritance between the GSC and the GB, a property that underlies that aforementioned necessity of proper spindle orientation in the balance of asymmetric cell division[13]. Specifically, the GSC inherits the mother centrosome, which retains position to the tip of the testis while the GB inherits the daughter centrosome, which displaces away from the tip[13]. Moreover, another cell intrinsic factor associated with centrosome positioning, proper spindle orientation, microtubule formation, and thus balance of GSC renewal is the localization of *Apc2* and *Apc1* proteins, *Drosophila* homologues of Adenomatous Polyposis Coli (APC) tumor suppressor gene that are required for many cytoskeletal signaling pathways[14,15]. Perturbations to spindle orientation and centrosome positioning was observed in *Apc2* and *Apc1* mutants accompanied by phenotypic defects[11].

Home base: the niche

Extrinsic and intrinsic methods of maintaining proper stem cell renewal and differentiation converge in the niche. The niche is a local microenvironment that provides "instructions" for regulation of stem cell renewal through various signaling pathways. Due to the morphogenic nature of many signaling pathways, physical proximity to the niche is important in maintaining stemness or inducing differentiation through extrinsic means. In the male *Drosophila* germline, a cluster of niche cells form the hub, which is anchored and kept at the tip of the testis (Figure 2A) through cell adhesion proteins such as integrins[16,23]. The hub physically touches around 10 GSCs that are arranged concentrically around the niche cells and only the

cells that are touching the hub are able to receive stemness renewing signals (Figure 2B). Hub cells secrete the JAK/STAT ligand *upd*[4,5], which is the signal required for stem cell renewal by reinforcing GSC adhesion to the hub[17,21]. The *upd* ligand remains closely associated with the extracellular matrix after its secretion, thus preventing it from diffusing too far from the hub[18]. Cells that lose contact with the hub thus do not receive enough stemness renewal signals[19] and quickly initiate their differentiation program.

In order to maintain the extrinsic mechanisms influencing stem cell renewal, the GSC and GB must keep up with their specific positioning relative to the hub and this may be achieved through intrinsic means, specifically by the aforementioned specific microtubule spindle formation[11,13]. The perpendicular orientation of the GSC toward the tip of the testis[11] is maintained during stem cell renewal because this ensures that the GSC is touching the hub and able to receive the signals dictating renewal. In order to maintain the position of the GSC adjacent to the hub, displace the GB away from the hub, and prevent competition[20] with the GSC for physically touching the hub, the cells must be divided in a manner that retains these properties: this is achieved by the previously mentioned cell intrinsic asymmetric centrosome inheritance in the GSC and the GB[13]. The GSC inherits the mother centrosome, which remains tethered to the GSC-hub border, while the GB inherits the daughter centrosome which moves away from the hub. The mother and daughter centrosome in turn display differences amongst themselves: the mother centrosome preserves much more robust interphase microtubules and is associated with many more microtubules through the cell cycle than the daughter centrosome[13]. This property of the mother centrosome may play a role in anchoring the GSC to the hub and preserve stemness, while the fewer microtubule associated daughter centrosome may allow the GB more freedom to move away from the hub and initiate differentiation[22].

Epigenetics and Influence on Asymmetric Cell Division

The asymmetric localization of cell intrinsic factors in the GSC and the GB that promote the maintenance of the balance between stem cell renewal and differentiation even extends to the epigenome[24]. Epigenetics refer to the inherited gene expression and resulting phenotypic changes that do not involve changes in the actual DNA sequence[25]. One of the methods in which similar DNA sequences yield varying resulting gene expression is through histones and the effect histone modifications have on the chromatin environment[26,27]. The fundamental unit of chromatin in eukaryotes is the nucleosome, a structure that consists of two H2A-H2B dimers and one H3-H4 tetramer that form a histone octamer which the DNA wraps around (Figure 1B). The histone proteins have tails, which are the sites of various modifications that influence chromatin environment and thus gene expression[27]. For example, methylation of histone tails often results in compaction of the chromatin and thus difficulty for transcriptional machinery to access the DNA, resulting in decreased gene expression. Thus, histones and histone modifications are major carriers of epigenetic modification as varying modifications on identical gene sequences can yield different results[19]. When a cell divides, all of components, including histone proteins, are replicated and segregated into the resulting daughters. In the male *Drosophila* germline, the GSC inherits most of the old (canonical) histone H3 while the GB inherits most of the new (newly synthesized) histone H3[23]. This asymmetric segregation of intrinsic factor is necessary for proper asymmetric cell division as this histone inheritance asymmetry was not observed in symmetrically dividing GBs[23].

Sequential Loading of Proteins Required for DNA Replication Initiation

Due to the effect that histone modifications have on gene expression and the interplay between gene expression and various cell cycle events[28,29], it can be reasoned that asymmetric

histone inheritance within the GSC and GB can affect critical cell cycle events such as that of DNA replication initiation in the epigenetically different cells. DNA replication begins with the separation of the dsDNA molecules into two single stranded molecules forming a replication bubble. Each replication bubble contains two replication forks, one at each side of the bubble. Due to the directionality of DNA and the ability of DNA polymerase to only add bases to the 3' end, each replication fork contains a leading strand and a lagging strand of strand elongation. The leading strand is continuously synthesized, but due to directionality of DNA and DNA polymerase activity, the lagging strand is synthesized in Okazaki fragments that are then ligated[30].

To start the process of DNA replication initiation, DNA replication initiation events begin during late mitosis of the previous cell division. After the separation of the sister chromatids, in late mitosis and G1 phase, the origin of replication complex (Orc1-6) binds to DNA[31]. The site of ATP-dependent Orc binding[31] is rather non-specific[32], but in Drosophila have often been seen to be near DNA replication start sites[33] and transcription start sites[34]. Orc then recruits cell division cycle 6 (Cdc6)[35], so that the mini-chromosome maintenance 2-7 (MCM2-7) chromatin licensing and DNA replication factor 1 (Cdt1) complex can be recruited to DNA[36] (Figure 3). MCM2-7 is the helicase that unwinds the DNA double helix for DNA replication to begin[37] while Cdt1 is a protein that ensures that DNA replicates only replicates once per cycle[38]. From here, signaling from cell dependent kinases (Cdks) and Dbf4 dependent kinases (Ddks) induce firing of the replication forks to drive the G1 to S transition[39,40] by activating MCM via phosphorylation[41] and inducing binding of Cdc45, a protein that must be recruited for proper helicase function[42,43,44]. At this point, the GINS complex along with DNA polymerases are loaded and the Cdc45, MCM2-7, and GINS complex form the CMG complex (named from the

acronym of the Cdc45,MCM2-7, and GINS complex) to allow for the progression of the replication fork and thus DNA replication[45]. As DNA replication occurs, polymerase activity is greatly enhanced by stabilization of the polymerase with the DNA via association with DNA clamps such as proliferating cell nuclear antigen (PCNA)[46], making PCNA an integral protein recruited during initiation of DNA replication.

Implications of Understanding Asymmetric Cell Division

The details of the physical and molecular differences resulting in drastically different fates of cells that arise from the same progenitor is still not completely understood. We are still figuring out how and what various biological mechanisms are utilized to maintain this delicate balance between stem cell renewal and differentiation to keep an organism in perfectly functional condition. Furthermore, it is still unclear what specific cellular processes diverge as a result of these different cell fates and what mechanisms within these cells are responsible for observed phenomenon.

Stem cells that are capable of asymmetric cell division are remarkable in that they are not only capable of renewing themselves and but can also replenish depleted differentiated daughter cells. The necessity of understanding the details of how this process is achieved and balanced is two-fold: elucidating stem cell renewal can bring to light foundations of cancer and potential ways stem cells can be used as therapy in treating symptoms of senescence and aging.

Chapter 2: Asymmetry of DNA Replication Initiation

Introduction

Asymmetric cell division yields two distinct daughter cells that both contribute significantly to the proper maintenance of an organism over time. Though the genetic sequence of these two cells is nearly identical, the respective cell fates and properties are drastically different and retained throughout life. Thus, it is logical to infer that that these two daughter cells must differ in some way that contributes to their marked characteristic, namely their epigenomes. Using a two-color system to visualize old and new histone in the male *Drosophila* germline, we observed that the GSC is enriched with old H3 while the GB is enriched with new H3[24]: asymmetric cell division may be required for GSCs to retain old histones and thus their stemness program. Furthermore, it has been observed that this asymmetric histone segregation is actually asymmetric at the level of single DNA strands[47]. A two-step model has been proposed to explain old and new histone enrichment patterns: first, during DNA replication, the leading strand retains most of the old histones while the lagging strand retains most of the new histones. Second, during anaphase, the old histone enriched sister chromatid is segregated to the GSC while the new histone enriched sister chromatid is segregated to the GB[47] (Figure 4A-4B). Tentative reasoning behind this revolves around the different kinetics in which the lagging and leading strands are synthesized in DNA replication. Due to the directionality of DNA synthesis, it takes the lagging strand longer to synthesize than the leading strand because it is synthesized from Okazaki fragments that must be ligated. Thus, lagging strand DNA is not synthesized fast enough for the old histones to be deposited into, so the pre-existing histones are deposited on the already synthesized leading strand while the lagging strand is enriched with newly synthesized H3[47].

A distinguishing mark of old histone is Threonine 3 (Thr3) phosphorylation on H3 (H3T3P)[48]. When this Thr3 is mutated to an Alanine (H3T3A), phosphorylation is no longer possible and histone segregation in the GSC/GB pair is randomized, thus providing evidence for the requirement of H3T3P for proper sister chromatid segregation and asymmetric histone inheritance[48]. Moreover, the H3T3A mutation conferred major cellular defects in the early germline that were not observed in the late stage germ cells and somatic cells[48], supporting the idea that proper asymmetric histone segregation is essential for preservation of healthy GSC/GB balance.

The GSC and GB have differential histone condensation and resulting compaction[49]. Specifically, the GSC inherits more histones and has a more condensed histone inheritance than the GB and thus takes more time to decondense at the end of telophase than the GB[49]. The differential condensation may be a underlying factor as to why the GB has a nearly nonexistent G1 phase because the GB does not need as much time as the GSC to decondense its chromatin before entering the next cell cycle. A critical histone modification that was observed to be essential to this differential condensation and compaction is Serine 10 (Ser10) phosphorylation on H3 (H3S10P), modification that is highly specific to mitosis due to chromatin condensation during mitosis[50,51]. When Ser10 is mutated to an Alanine (H3S10A), differential condensation was lost in the GSC and GB[49]. Moreover, when male Drosophila germ cells were subjected to treatment to Nocodazole, a microtubule depolymerizer[52], histone asymmetry was also lost[49], bringing back to the importance of proper spindle formation and orientation in maintaining proper stem cell renewal[11,13].

With the various studies done on elucidating the methods in which proper epigenetic inheritance is maintained within the GSC and the GB, the next question of interest is what, if

any, are the immediate downstream effects of this observed asymmetric histone inheritance? We have observed that DNA replication initiation timing is asymmetric in the GSC and the GB, in which the GB enters DNA replication initiation faster than the GSC does. Moreover, we have also seen potential asymmetry in DNA replication initiation factors such as Cdc6 and Orc1 in the GSC and GB. Previous studies at the single molecule level have seen the Orc1 dependent loading of Cdc6 to the pre-replication complex[52]. However, we have observed an Orc1 independent loading of Cdc6 in the male *Drosophila* germline system, making us wonder if the canonical view of replication initiation is conserved in our model system. Because of the link between histones, gene expression, and cellular processes, we hypothesize that the observed asymmetric DNA replication initiation is a direct downstream effect of histone inheritance asymmetry, specifically the differential condensation and compaction conferred to the GSC and GB by asymmetric histone segregation.

Materials and Methods

Raising and Dissection

Drosophila lines were raised in 25˚C incubator. Dissections were performed using minimal CO_2 in Schneider's Drosophila Medium that was warmed in a 29˚C water bath.

Experiments that required heat shock used *Drosophila* raised in 25˚C incubator, heat shocked in 37˚C water bath for 1.5 hours, and then allowed to recover in a 29˚C incubator for 22-23 hours and then immediately dissected following the above protocol.

Immunostaining and EdU Incorporation

Immunofluorescence staining was done according to standard procedures explicated in previous experiments[24]. Primary antibodies utilized were mouse anti-H3s10p (1:5000 Abcam ab14955), mouse anti-Armadillo (1:200 DSHB N2 7A1), mouse anti-α-spectrin (1:50 DSHB 3A9), mouse anti-PCNA (1:100 Santa Cruz sc-56), rabbit anti-mKO (1:200 MBL PM051M), rabbit anti-H3K4me3 (1:200 Cell Signaling #9751S), rabbit anti-GFP (1:200 Abcam ab290), rabbit anti-H3K9me3 (Abcam ab9045), chicken anti-GFP (1:1000 Abcam ab13970), and chicken anti-mCherry (1:1000 Novis NBP2-25158).

EdU labeling to help elucidate GSC-GB pairs was performed via Click-iT EdU Alexa Fluor 647 Imaging Kit (Invitrogen C10640) according to instructions from the manufacturer. Testes were then incubated in 2μM EdU for 10 minutes at room temperature on the rotator. Testes were fixed immediately after with formaldehyde/PBST and incubated in primary antibodies overnight at 4˚C. Fluorophore conjugation to EdU was performed the next day using the manufacturer's instructions which was followed by secondary antibody incubation for 2 hours. All washes between the steps was done using 1x PBST. Testes were then mounted using

Vectashield imaging media (ZE0806). Imaging was done on the laser confocal microscopes Zeiss LSM 700 and the Leica Dmi8 at 63x oil magnification.

Nocodazole Treatment

For Nocodazole treatment arrest and release, testes were incubated in Nocodazole (1:300 dilution of NZ stock in imaging media+insulin) for 3-4 hours immediately after dissection with cap open for air exchange; Nocodazole solution was changed for fresh solution every hour. Testes were then washed for 15 minutes in Schneider's *Drosophila* Medium and fixed immediately after with formaldehyde/PBST. For experiments that required EdU incorporation, testes were then incubated in 2μM EdU for 10 minutes at room temperature on the rotator and then washed for 15 minutes in Schneider's *Drosophila* Medium. Testes were allowed to rest for 35 minutes to achieve desired cell cycle stage and then incubated in primary antibodies overnight at 4˚C, conjugated to fluorophores, and imaged according to aforementioned protocol.

Live Cell Microscopy

Testis were subjected to Nocodazole treatment according to aforementioned protocol and immediately transferred to an imaging chamber as described in Ranjan et. al, 2019[49] then imaged on Zeiss AxioObserver Yokogawa CSU-X1M Spinning Disk Confocal microscope in a heated chamber at 30˚C with controlled CO_2 flow. Images were collected ever 90 second for 3 hours.

Quantifications

Quantifications were done using imageJ software and graphed using Graphpad Prism 8. Values of proteins of interest were calculated using the ratio of GSC/GB and then graphed after taking $\log_2$ of the specific values.

Drosophila lines that were engineered for this project are as follows in Table 1 below.

Drosophila Lines Used		
Line	Source	Comments
PCNA-EGFP	A gift from the lab of Eric F. Wieschaus (59)	For the PCNA WT and Nocodazole, fixed and live, experiments
Hs-flip; Nanos-Gal 4	Bloomington Stock Center BL-26902	Heat shock flip early germline driver
UASp-H3V6	Transgene created in the Chen lab and then injected into Drosophila embryos at BestGene (24)	To reveal histone H3 for the H3 Nocodazole experiments
UASp-FRT-H3-GFP-PolyA-FRT-H3-mKO	Transgene created in the Chen lab and then injected into Drosophila embryos at BestGene	Scheme was paired with a heat-shock flip, nanos-Gal4 driver to reveal old and new histone H3 in the early germline (24)
UASp-FRT-H3-GFP-PolyA-FRT-H3T3A-mKO	Transgene created in the Chen lab and then injected into Drosophila embryos at BestGene	Scheme was paired with a heat-shock flip, nanos-Gal4 driver to create H3T3A mutant background (48)
UASp-FRT-H3-GFP-PolyA-FRT-H3S10A-mKO	Transgene created in the Chen lab (Ranjan) and then injected into Drosophila embryos at BestGene	Scheme was paired with a heat-shock flip, nanos-Gal4 driver to create H3S10A mutant background
Cdc6-HA	CRISPr engineered tag created by Chen Lab (Wooten and Snedeker) generated by FunGene and then injected into Drosophila embryos	Crossed with Orc1-GFP to create Cdc6-HA x Orc1-GFP line (Chu)
Cdc6-mcherry	CRISPr engineered tag created by Chen Lab (Wooten and Snedeker) generated by FunGene and then injected into Drosophila embryos	Crossed with Orc1-GFP to create Cdc6-mcherry x Orc1-GFP line (Chu)
Orc1-GFP	CRISPr engineered tag created by Chen Lab (Wooten and Snedeker) generated by FunGene and then injected into Drosophila embryos	Crossed with Cdc6-mcherry to create Cdc6-mcherry x Orc1-GFP line (Chu)
UASp-Orc5-mcherry	Transgene created in the Chen lab and then injected into Drosophila embryos at BestGene	For Orc5-mcherry experiments

Table 1: Table explicating which fly lines were used, their sources, and what the lines were specifically used for.

Results

In order to examine the hypothesis that DNA replication initiation timing asymmetries following mitosis are downstream of asymmetric histone inheritance, we visualized DNA replication initiation under conditions that randomize histone inheritance. By using PCNA or EdU as a marker of DNA synthesis and replication initiation, we examined the patterns of DNA replication initiation of the GSC and GB under two conditions that randomize histones, Nocodazole treatment and H3T3A mutation. In an effort to find a molecular mechanism for replication initiation dynamics, we also studied the dynamics of Cdc6 and Orc1 during mitosis in the male *Drosophila* germline. While the two factors showed inconsistent distributions in their loading, the timing of their loading could suggest a germline specific replication initiation program. The previous asymmetry in these replication factors that we have seen may potentially be a result of the properties resulting from asymmetric histone inheritance and in turn play a role in the observed phenomenon of DNA replication initiation asymmetry in the GSC and the GB.

Asymmetry of DNA Replication Initiation in the GSC and the GB

Using either EdU, a thymidine analog, or PCNA, an essential cofactor for DNA polymerase that must be recruited to the replication fork at the onset of DNA replication[52], as indications of active DNA replication, we can visually study the progression of DNA replication. At the onset of DNA replication initiation, we previously observed that there exists an asymmetry in DNA replication initiation timing in the asymmetric GSC/GB division, as indicated by the PCNA segregated towards the GB compared to the GSC (Figure 5A). However, this DNA replication initiation asymmetry is not observed in the symmetric GB/GB division, as shown by the symmetric distribution of PCNA between the GBs (Figure 5B). Moreover, there is a nearly non-existent G1 phase in the GB as shown by absence of nucleolar reformation, a key

G1 cell cycle process[54]. This is indicated by the lack of Fibrillarin, a protein that is a marker for the site of nucleolar formation[53], in the GB prior to replication entry (Figure 5B). On the other hand, the GSC does have a G1 phase and takes time to reform its nucleolus before the onset of S phase (Figure 5A). Together, these observations provide evidence that the GB enters DNA replication and the next cell cycle faster than the GSC does.

Nocodazole, Randomized Histone Inheritance, and DNA Replication Initiation

Asymmetric histone segregation is necessary for proper asymmetric cell division maintenance[24] and disruption of this asymmetric inheritance leads to phenotypic defects in the germline[48]. With the observation that the GB enters DNA replication before the GSC in the normal asymmetrical dividing GSC/GB pairs in the wild type *Drosophila* germline, we hypothesized that there may exist a link between asymmetric histone inheritance and observed asymmetric entry into DNA replication initiation. Previous studies showed that proper stem cell renewal requires specific spindle orientation[11], centrosome inheritance[13], and microtubule dynamics[11] and that utilizing Nocodazole, a microtubule depolymerizer, randomizes asymmetric microtubule activity in GSCs and results in symmetric histone inheritance between the GSC and GB[49]. When we utilized Nocodazole treatment (Figure 6A), we not only confirmed that symmetric histone inheritance resulted from Nocodazole treatment but also observed globally symmetric entry into DNA replication initiation as indicated by the equal segregation of PCNA in the GSC and GB (Figure 6B). The strict enrichment of old histone H3 towards the GSC and new H3 towards the GB is eliminated[24] as is the observed traditional asymmetry of DNA replication initiation with the GB beginning this process before the GSC does.

Due to both the randomized histone inheritance induced by Nocodazole treatment along with the utility of the drug to enhance catching our desired timepoint by arresting, releasing, and

waiting a specific time before fixing (Figure 6A), we were able to greatly enrich for cells during DNA replication initiation to more efficiently study the patterns of DNA replication initiation in the germline. Subjecting testis to Nocodazole treatment resulted in DNA replication entry randomization. We observed all three possible temporal patterns of replication timing, including traditional DNA replication initiation asymmetry where the GB enters DNA replication prior to the GSC as seen from enrichment of PCNA towards the GB (Figure 7A), symmetric DNA replication initiation where the GB and GSC enter DNA replication at the same time as seen from equal enrichment of PCNA in the GSC and GB (Figure 7B), and reverse DNA replication initiation asymmetry with the GSC entering DNA replication prior to the GB as indicated from the enrichment of PCNA towards the GSC (Figure 7C). We restrict our analysis of cells entering DNA replication to cells where the heterochromatin is still S10P positive and replication factors are enriched in the euchromatin, consistent with the idea that the cells recently divided and are now just entering DNA replication. To confirm our observations, we observed DNA replication initiation after Nocodazole release *in vivo* under live cell microscopy and we observed cells pairs with reverse asymmetric entrance into DNA replication (Figure 7D, t=6s) as seen from the brief enrichment of PCNA towards the GSC before the GB also begins DNA replication. Quantifications of PCNA enrichment in the GSC and the GB following Nocodazole arrest and release resulted in complete randomization of entry to DNA replication initiation. Most entries became symmetric, as seen from the cluster near 0, though we also observed some cases of traditional asymmetric entrance to DNA replication, PCNA enrichment towards the GB, and various cases of reverse asymmetric entrance to DNA replication, PCNA enrichment towards the GSC. In summary, the effects of Nocodazole treatment randomized DNA replication initiation asymmetry in the GSC and GB: the consistent trend of the GB entering DNA replication

initiation prior to the GSC is eliminated and we observe all types of different entries to DNA replication under these conditions.

Randomized Histone Inheritance and DNA Replication Initiation Asymmetry

Due to the effect that Nocodazole has on microtubule dynamics, this symmetric DNA replication entry may be due to either the effect of Nocodazole on microtubules and the loss of some cytoplasmic determinant or due to the resulting downstream symmetric histone inheritance. To elucidate whether the observed non-canonical pattern of DNA replication initiation is downstream of histone inheritance or an effect of microtubule perturbations, we looked at DNA replication initiation in a randomized histone mutant background, H3T3A. H3T3 phosphorylation is a distinguishing mark of old histone and necessary for proper sister chromatid segregation and asymmetric histone inheritance[48]: the H3T3A mutation prevents phosphorylation and is a microtubule-independent method of disrupting histone inheritance[48]. When looking at the pattern of DNA replication initiation in the H3T3A mutant background, we saw randomization of entry into DNA replication initiation. We observed traditional DNA replication initiation asymmetry with the GB entering DNA replication prior to the GSC (Figure 8A), symmetric DNA replication initiation with the GB and GSC entering DNA replication at the same time (Figure 8B), and reverse DNA replication initiation asymmetry with the GSC entering DNA replication prior to the GB (Figure 8C). All these conclusions used EdU, a thymidine analog only incorporated during S phase, as a marker for DNA replication and utilized mitotic specific H3S10P[50,51] to determine cell stage and to ensure we were looking at GSC/GB pairs. Quantification of EdU in the GSC and the GB showed a general symmetric entrance into DNA replication as shown from the wide collection of data near 0. However, as with the results from the Nocodazole arrest and release experiments, there were also cases of traditional asymmetry

and reverse asymmetry of DNA replication initiation in the GSC and the GB. These studies concluded that the randomization of DNA replication initiation observed after Nocodazole treatment was due to the randomization of histones rather than microtubule perturbations as we saw the same DNA replication entry randomization in the microtubule independent randomized histone mutant background. In summary, asymmetry of DNA replication initiation is likely a downstream effect of asymmetric histone inheritance.

The Timing of Loading of Replication Initiation Factors

The recruitment of various factors such as Cdc6 and Orc1 are essential for the activation of DNA replication[33,36]. Varying chromatin environments in the GSC and GB may affect the timing of recruitment of replication initiation factors such as Cdc6 and Orc1. We have preliminary evidence of the potential asymmetry of the loading of these DNA replication initiation factors. We have previously seen an asymmetry of loading of Cdc6 in which there is an enrichment of Cd6 towards the GB. This may be a result of the more open and less condensed mitotic chromatin of the GB being able to load Cdc6 faster than the more condensed mitotic chromatin of the GSC. However, we have also observed a symmetric loading of Cdc6 (Figure 9B), implicating conflicting data of this loading. Additionally, we have observed deviations from the canonical view of loading of replication initiation factors in which Cdc6 recruitment is dependent upon Orc1 loading[57], Orc binds chromatin prior to Cdc6. However, in the male *Drosophila* germline, we have observed Cdc6 unloading prior to Orc1 and an independence of Cdc6 and Orc1 binding (Figure 9A). The temporal pattern of Cdc6 binding suggests further peculiarities in DNA replication initiation in our model system as we have seen that Cdc6 is sufficient to bind chromatin prior to Orc loading. Additionally, it is shown that Cdc6 has no association with DNA during prophase but an extremely strong association with DNA in

metaphase (Figure 9A), implicating that Cdc6 moves to the nucleus at some point during prometaphase. Following the canonical view of loading of replication initiation factors[57], we would also expect to see Orc loading at some point near prometaphase when Cdc6 is seen to first load, but this is clearly not the case as evidenced by the complete lack of Orc and DNA association in late prophase (Figure 9C). In fact, there is no association of Orc and DNA in late telophase or telophase (Figure 9C), indicating that Orc does not bind to DNA in the prior mitosis either.

Discussion

Asymmetric histone inheritance has proved to be a key cell intrinsic factor[24] upkeeping proper stem cell asymmetric cell division and sustaining the health of the male *Drosophila* germline[24,48], but there is a lack of exploration on the direct downstream effects of histone asymmetry in the GSC and GB. From the studies performed here, we have gathered evidence that DNA replication initiation, a crucial cellular event, is one of the major processes affected by asymmetric histone segregation. When observing DNA replication initiation in the wild type male *Drosophila* germline in both fixed wholemount and *in vivo*, we consistently see that the GB begins DNA replication before the GSC does. The localization of DNA replication factor PCNA toward the GB in asymmetric GSC/GB division along with the absence of G1 phase in the GB (Figure 5A), evidences that the GB seems to move through the cell cycle at a faster rate than the GSC. Thus, the GB begins cell cycle dependent processes such as DNA replication before the GSC begins.

Nocodazole is a microtubule de-polymerizer that also happens to eliminate the microtubule asymmetry that exists to maintain faithful asymmetric histone segregation and asymmetric cell division[49]. This drug is also very useful in enriching for the time point that we want to make conclusions about DNA replication initiation entry, late telophase/early G1/S. In the male *Drosophila* germline, there are only around 10 GSCs and when doing experiments, we catch only around 2 GSCs actively undergoing mitosis per testis as the rest of the cells are in G2, the longest phase of the cell cycle in the germline. Thus, it is difficult to observe entry to S-phase/DNA replication initiation with high frequency. Therefore, Nocodazole arrest and release greatly enhances catching our desired time point and also allows us to view DNA replication initiation patterns in the GSC and GB when histones are randomized (Figure 6A). When

subjecting the germline to Nocodazole treatment, not only was asymmetric histone segregation randomized, so was DNA replication initiation asymmetry (Figure 6B). From this initial observation, we further investigated DNA replication initiation to gather more data about replication initiation randomization. By subjecting flies of the PCNA-EGFP line with Nocodazole arrest and release followed by a pulse of EdU and chase, we were able to more conclusively recapitulate our previous observation that DNA replication initiation was randomized following Nocodazole treatment. (Figure 7A-C). To further confirm our observation, we observed DNA replication initiation using live cell imaging after Nocodazole arrest and release and saw randomization of DNA replication initiation *in vivo* (Figure 7D).

We hypothesized that the randomization of DNA replication initiation is a downstream effect of randomized histone asymmetric, but we could not rule out the potential that the replication initiation randomization was an effect of Nocodazole's effect on microtubule polymerization. Thus, we had to observe DNA replication initiation in a randomized histone background that was not achieved through direct microtubule perturbations. Therefore, we decided to conduct our experiments in the H3T3A mutants. When observing replication initiation patterns in an H3T3A mutant background, a microtubule independent asymmetric histone randomization, we also observed randomization of initiation of DNA replication, thus confirming our hypothesis that DNA replication initiation asymmetry is downstream of asymmetric histone inheritance (Figure 8A-C). When traditional histone asymmetry is broken, DNA replication initiation asymmetry is no longer faithfully reproduced.

Due to the asymmetric histones inherited by the GSC and GB[24], the effect of the varying epigenomes must contribute to different chromosome landscapes between the two cells. A more open chromatin environment and less condensation/compaction, such as that in the GB,

physically allow for easier and faster recruitment of proteins while a more closed chromatin environment needs more time to de-condense and recruit proteins, such as that in the GSC[56]. These factors may play a role in recruiting proteins necessary for DNA replication initiation, namely Cdc6 and Orc1. We have previously observed an instance where there was asymmetry in Cdc6 loading in the GSC and GB in which enrichment was towards the GB, but symmetric Cdc6 loading has also been observed (Figure 9B). Furthermore, we have also previously seen an asymmetry in loading of Orc1 where there is an enrichment of Orc1 towards the GB and only a dot of Orc1 observed in the GSC. This observation may also be a result of the different chromatin environments in the GSC and GB due to asymmetrical histone inheritance induced differential condensation and compaction: the GB is able to recruit more Orc1 while the GSC is so much more compacted that it cannot load as much, resulting in the observed dot. The lack of unity between our observations of DNA replication initiation factors loading with the canonical view of replication initiation loading further asks us to question whether this canonical loading is conserved in the *Drosophila* male germline. Cdc6 is recruited by Orc prior to DNA replication initiation, but we have not yet seen a definitive point in which both the proteins are recruited to DNA together (Figure 9A). Cdc6 is seen to begin strongly associated with DNA during metaphase and completely excluded from DNA in prophase (Figure 9A), implying that it becomes recruited some time in late prometaphase (Figure 9A). In order for our observations and the canonical view of loading of replication initiation factors to unite, we would expect Orc to bind to chromatin at some point during prometaphase so that it can recruit Cdc6, but that has not been observed (Figure 9A). Orc was also not seen to bind in prophase (Figure 9C), or in telophase or late-telophase (Figure 9C), implying that it is not loaded in the phase prior to prometaphase and also is not loaded at the end of the prior mitotic cycle. Thus, our observations

show that not only does Cdc6 seem to be able to be loaded to chromatin independently of Orc, but that there may also be asymmetry in the loading of these replication factors in the GSC and the GB. Further experiments are underway to more clearly observe and understand the discrepancy from the canonical view and what we observe of replication initiation factor loading in our model system along with potential asymmetries in this loading between the GSC and GB in the *Drosophila* germline.

In summary, we have concluded that asymmetry of DNA replication initiation is a downstream effect of asymmetric histone inheritance. For the first time, we have observed that there exists a distinct cell cycle difference in the GSC and GB due to histone asymmetry segregation in the male *Drosophila* germline. A combination of structural and molecular factors may explain our observations: the differing chromatin landscapes conferred to the GSC and GB by asymmetric histone inheritance affects the proceeding of cell cycle events in these cells and may in part also affect the recruitment of various proteins needed for these processes. Gene expression is also likely differentially regulated in these cells and contribute to the observed asymmetric in cell cycle processes in these two cells. These differences illustrate that cell intrinsic factors immediately reprogram the two cells following division, cementing the fates the GSC and the GB. The GSC is the self-renewing stem cell that will continue to remain a stem cell. This is the cell that consistently is enriched with old histones, perhaps retaining the old histones to "remember" its program on how to renew its stemness. On the other hand, the GB is the differentiating daughter cell that goes on to become sperm and contribute its genetic information to the next generation. It is the cell that inherits the new histones, which potentially contains instructions on initiating its differentiation program.

Future Directions

One of the major effects of asymmetric histone inheritance is differential condensation and compaction of chromatin in the GSC and GB, specifically that the GB inherits less histone and thus has lesser resulting condensation and compaction of chromatin[49]. Prior observations have showed that the GB inherits the new histones and has less histone compared to the GSC and the GB also has decreased condensation and compaction[49]. The decrease in condensation explains its quicker entrance to the next cell cycle due to the less time needed for chromosome de-condensation in telophase and absence of a G1 phase, both of which may serve to inhibit DNA replication initiation[55]. The decreased compaction from fewer histone and possibly histone modification asymmetries create a more open and accessible chromatin environment[26]. The GSC on the other hand inherits markedly more old histone, and requires more time to fully condense and de-condense through mitosis along with a more closed chromatin environment[49], potentially explaining its temporal delay in entering the next cell cycle when compared to the GB. We hypothesize that this asymmetric entrance into DNA replication may be in part due to the differential mitotic condensation conferred by asymmetric histone inheritance. To test our hypothesis, we are currently observing DNA replication patterns in the H3S10A mutant background, a mutation that eliminates differential condensation[49]. In this background, if we see symmetric entry of DNA replication in the GSC and GB, using EdU as a marker for S phase and S10P as a confirmation that we are observing early DNA replication, we can conclude that asymmetry in DNA replication initiation timing is an effect of the differential condensation that normally exists in the GSC and GB due to asymmetric histone segregation. The effect of histone asymmetry on the differential condensation/compaction in the GSC and GB may also affect the

timing of loading of replication initiation factors that in turn contribute to the observed asymmetry of DNA replication initiation timing.

We also want to continue observing patterns of Cdc6 and Orc binding in mitosis to elucidate whether the male *Drosophila* germline has a germline specific process of replication initiation factor loading. Despite the fact that single molecule studies have observed the canonical view of the necessity of Orc loading to recruit Cdc6[57], there have been studies done *in vivo* showing that in *C. elegans*, Cdc6 binds chromatin independently of Orc[58]. In order to more definitively observe replication initiation factor loading in our system, we are observing the patterns of Cdc6 localization under live microscopy. By doing this, we will not only be able to see Cdc6 enrichment through the cell cycle but also find out whether there truly is asymmetry in Cdc6 loading between the GSC and GB.

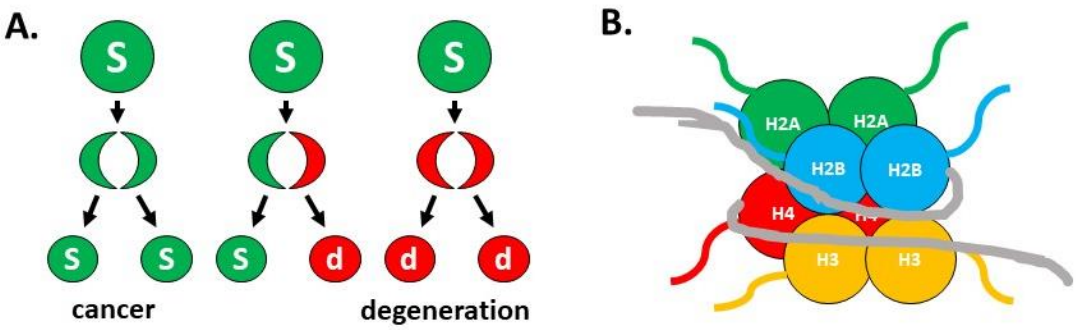


Figure 1: Asymmetric Cell Division and the Histone Octamer. (A) A model showing asymmetric cell division and consequences of stem cell (s) over-proliferation and stem cell over-differentiation (d). **(B)** A cartoon characterizing the histone octamer model consisting of two H2A-H2B dimers and one H3-H4 tetramer of which DNA (grey) wraps around.

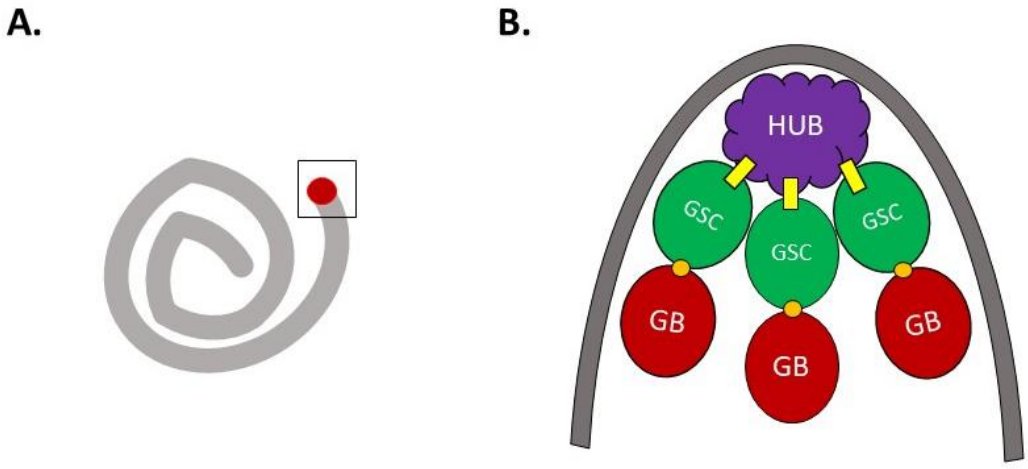


Figure 2: The Male *Drosophilia* Testis and the Stem Cell Niche. (A) An illustration showing the morphology of a male *Drosophila* testis. **(B)** A model showing en enlargment of the location of the stem cell niche at the tip of the testis (boxed). At the apical tip is the stem cell niche or hub, with the GSC (green) physically touching the hub and the GB (red) displaced perpindicularly away. GSC adheres to the hub via integrins (bright yellow rectangles) and the GSC/GB pair are physically connected via the spectrosome (orange circle).

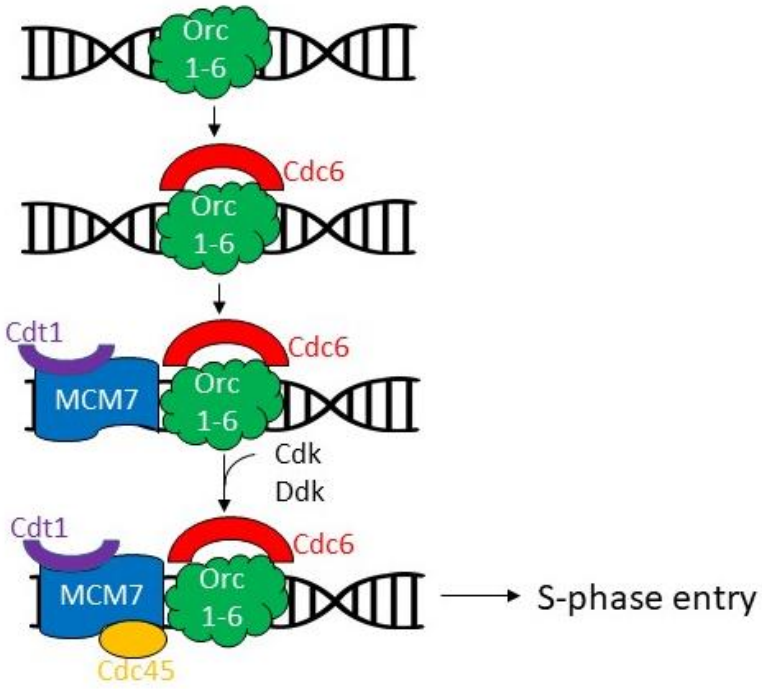


Figure 3: Loading of DNA Replication Initiation Factors. A model showing the sequential loading of replication factors during late telophase/early G1 phase that are required for successful entrance to S-phase and initiation of DNA replication.

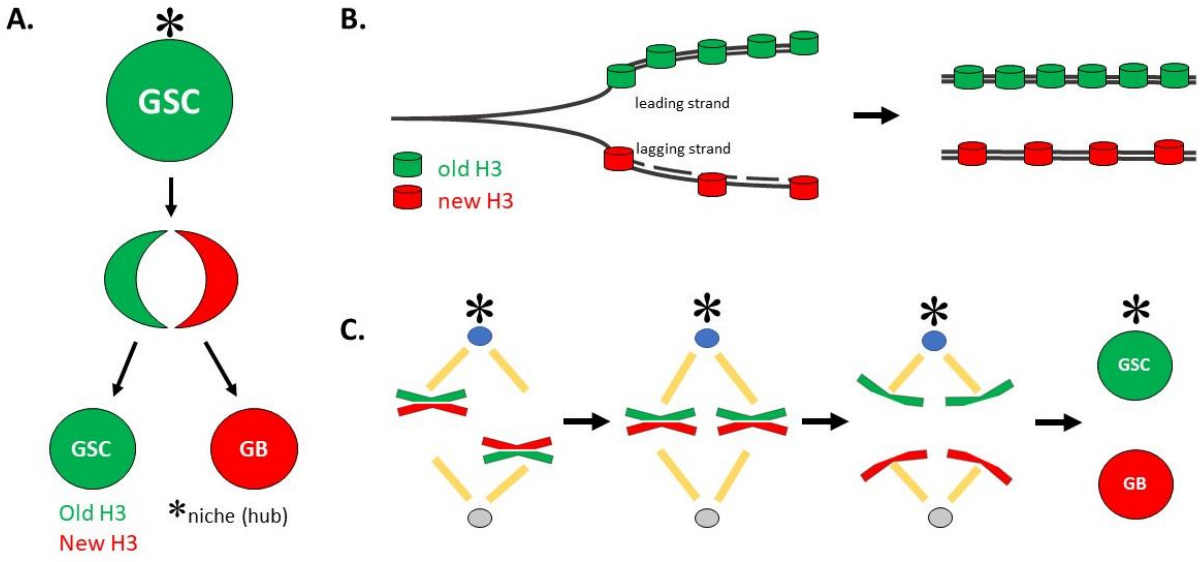


Figure 4: Proposed mechanism of asymmetric histone inheritance. (A) A model explicating the pattern of asymmetric histone inheritance in the GSC and the GB. The * symbolizes the hub and is used in all future models to indicate which cell is the GSC and which is the GB. **(B)** A schematic elaborating on step 1 of the two-step model of asymmetric histone inheritance in which the leading strand is enriched with old histone and the lagging strand is enriched with new histone. **(C)** A schematic elaborating on step 2 of the two-step model of asymmetric histone inheritance in which sister chromatids are selectively segregated by the microtubule machinery in mitosis such that the old histone enriched sister chromatids are segregated to the GSC while the new histone enriched sister chromatids are segregated to the future GB.

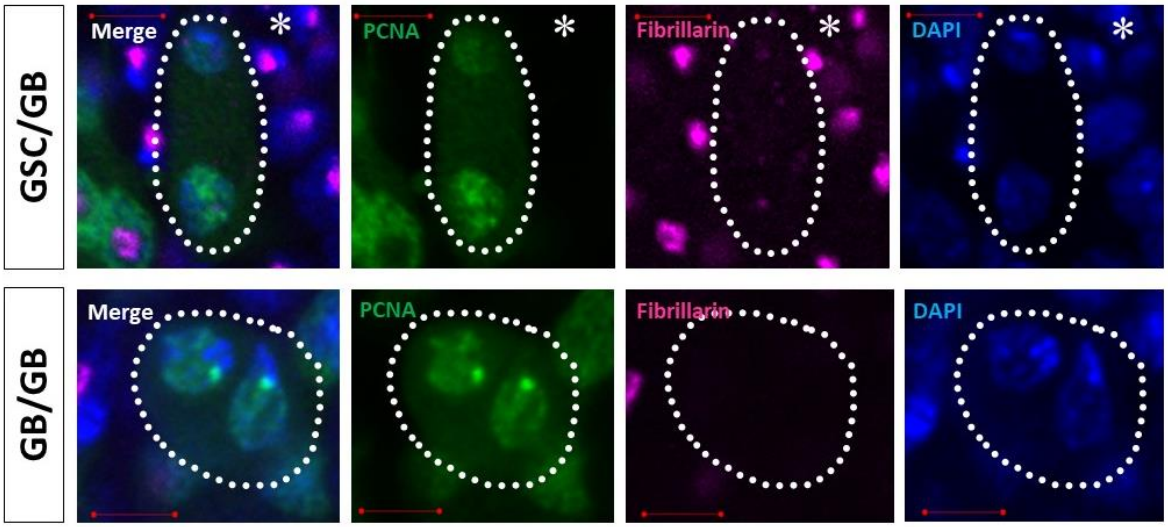


Figure 5: DNA Replication Initiation is Asymmetric in the GSC/GB Division. An asymmetric GSC/GB division shows the asymmetry in DNA replication initiation indicated by PCNA-GFP (green) enrichment towards the GB. A symmetric GB/GB division shows symmetry of DNA replication initiation indicated by equal segregation of PCNA-GFP (green) in the two cells. *=hub, scale bar is 5µm

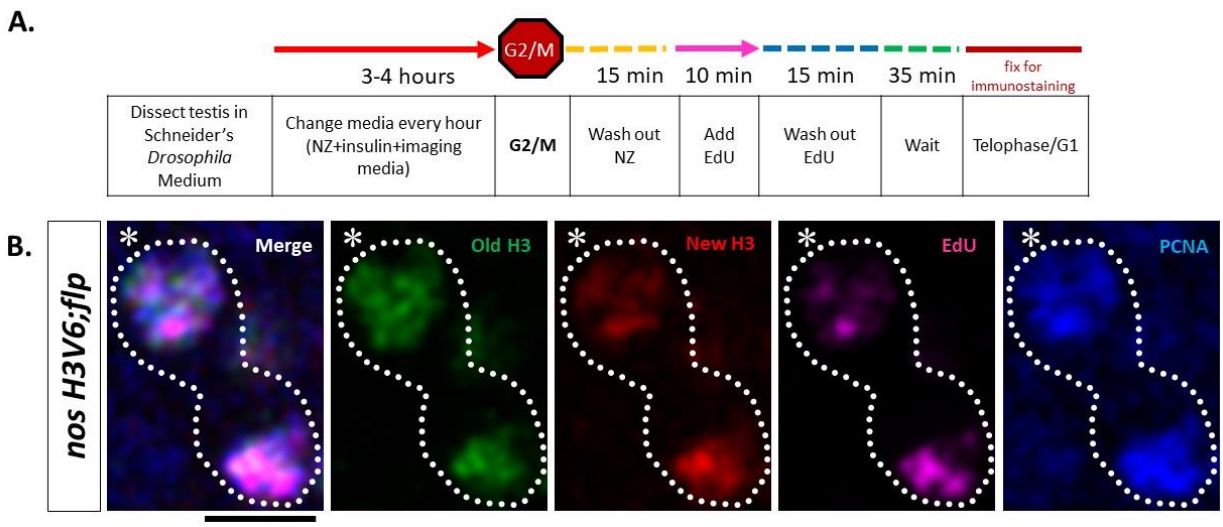
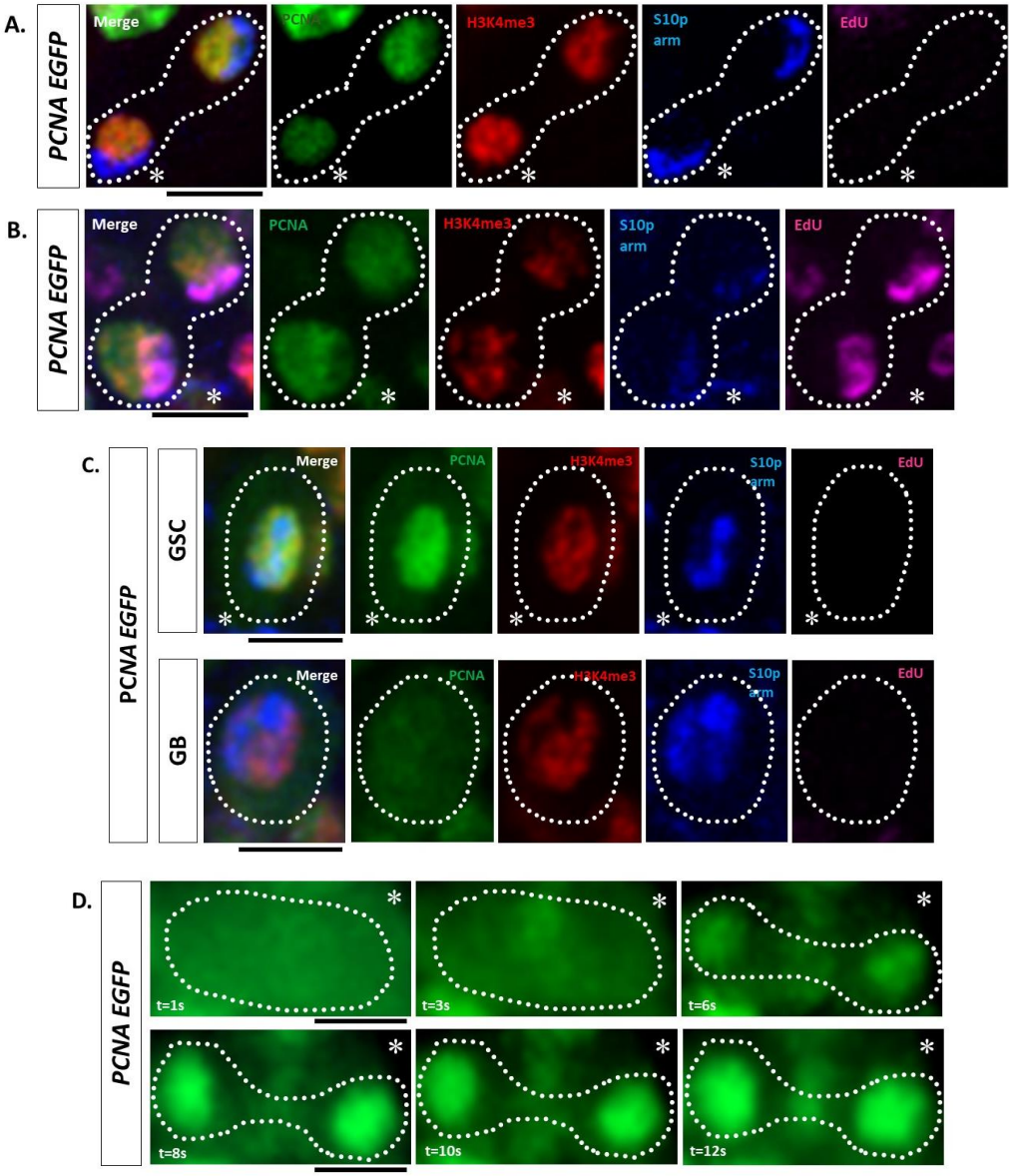


Figure 6: Nocodazole Protocol and Its Randomization of Histone and DNA Replication Initiation Asymmetry. **(A)** A schematic of Nocodazole-EdU pulse chase treatment experiment where testes were treated with Nocodazole for 3-4 hours to arrest in G2/M phase. Immediately after washing out to release arrest, EdU was incorporated to label S-phase and then washed out to remove any EdU not incorporated during DNA replication. This was followed by a 35 minute wait so that GSCs could progress to telophase/G1 and then fixed. **(B)** When treated with Nocodazole, asymmetric histone segregation was eliminated as seen from the symmetric old H3-GFP (green) and new H3-mKO (red) in the GSC and GB along with loss of DNA replication initiation symmetry as indicated by the symmetric enrichment of PCNA (blue) in the GSC and GB. *=hub, scale bar is 5µm


A.
PCNA EGFP
Merge
PCNA
H3K4me3
S10p arm
EdU
B.
PCNA EGFP
Merge
PCNA
H3K4me3
S10p arm
EdU
C.
PCNA EGFP
GSC
GB
Merge
PCNA
H3K4me3
S10p arm
EdU
D.
PCNA EGFP
t=1s
t=3s
t=6s
t=8s
t=10s
t=12s

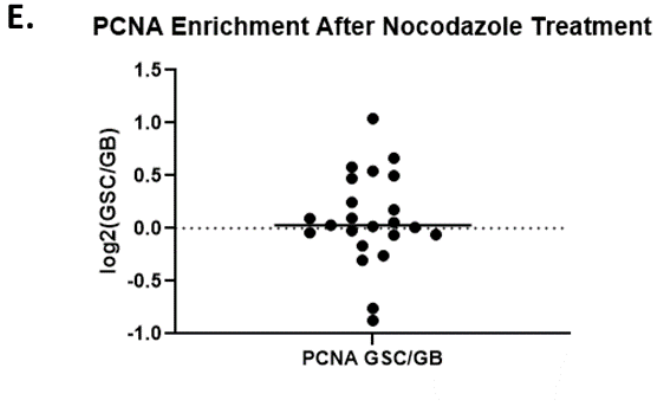

E.
PCNA Enrichment After Nocodazole Treatment
log2(GSC/GB)
1.5
1.0
0.5
0.0
-0.5
-1.0
PCNA GSC/GB

Figure 7: Nocodazole Randomizes DNA Replication Initiation. (A) Traditional asymmetry, PCNA-GFP (green) enriched towards GB, **(B)** symmetry, symmetric PCNA-GFP (green) enrichment, and **(C)** reverse asymmetry, PCNA-GFP (green) enriched towards GSC of DNA replication initiation when testis are treated with Nocodazole. **(D)** Live cell imaging of Nocodazole arrested and released cells showing reverse asymmetry of entry in DNA replication initiation as seen from PCNA-GFP (green) enrichment towards the GSC at t=6. **(E)** Quantification of the ratio of PCNA-GFP (y:axis $\log_2$ scale) fluorescence intensity in GSC-GB pairs. *=hub, scale bar is 5μm

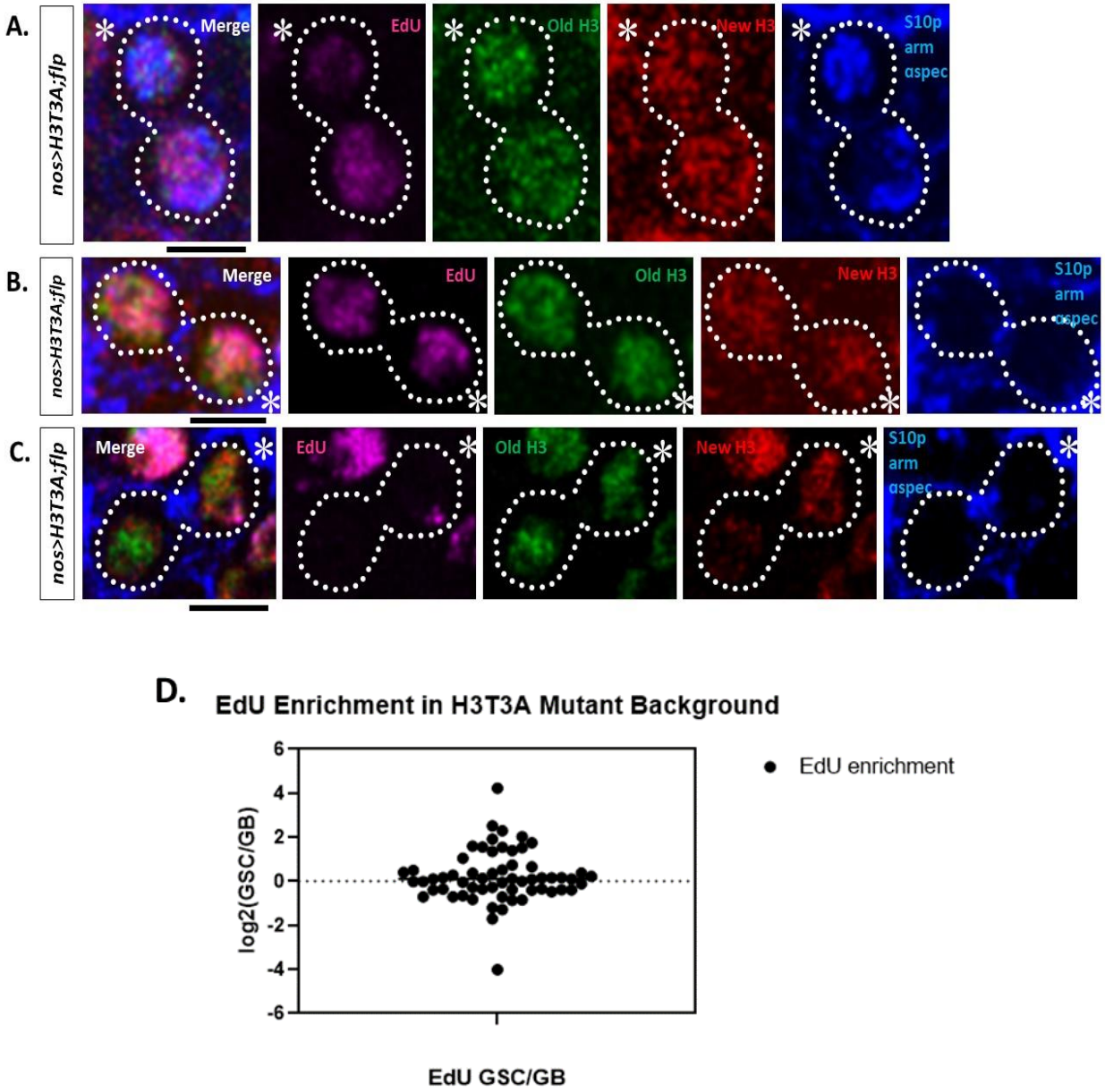


Figure 8: DNA Replication Initiation Asymmetry is Randomized in a H3T3A Mutant Background. (A) Traditional asymmetry (EdU enrichment towards the GB), **(B)** symmetry (symmetric EdU enrichment between the GSC and GB), **(C)** reverse asymmetry (EdU enrichment towards the GSC). **(D)** Quantification of the ratio of EdU (y:axis $\log_2$ scale) fluorescence intensity in GSC-GB pairs. *=hub, scale bar is 5µm

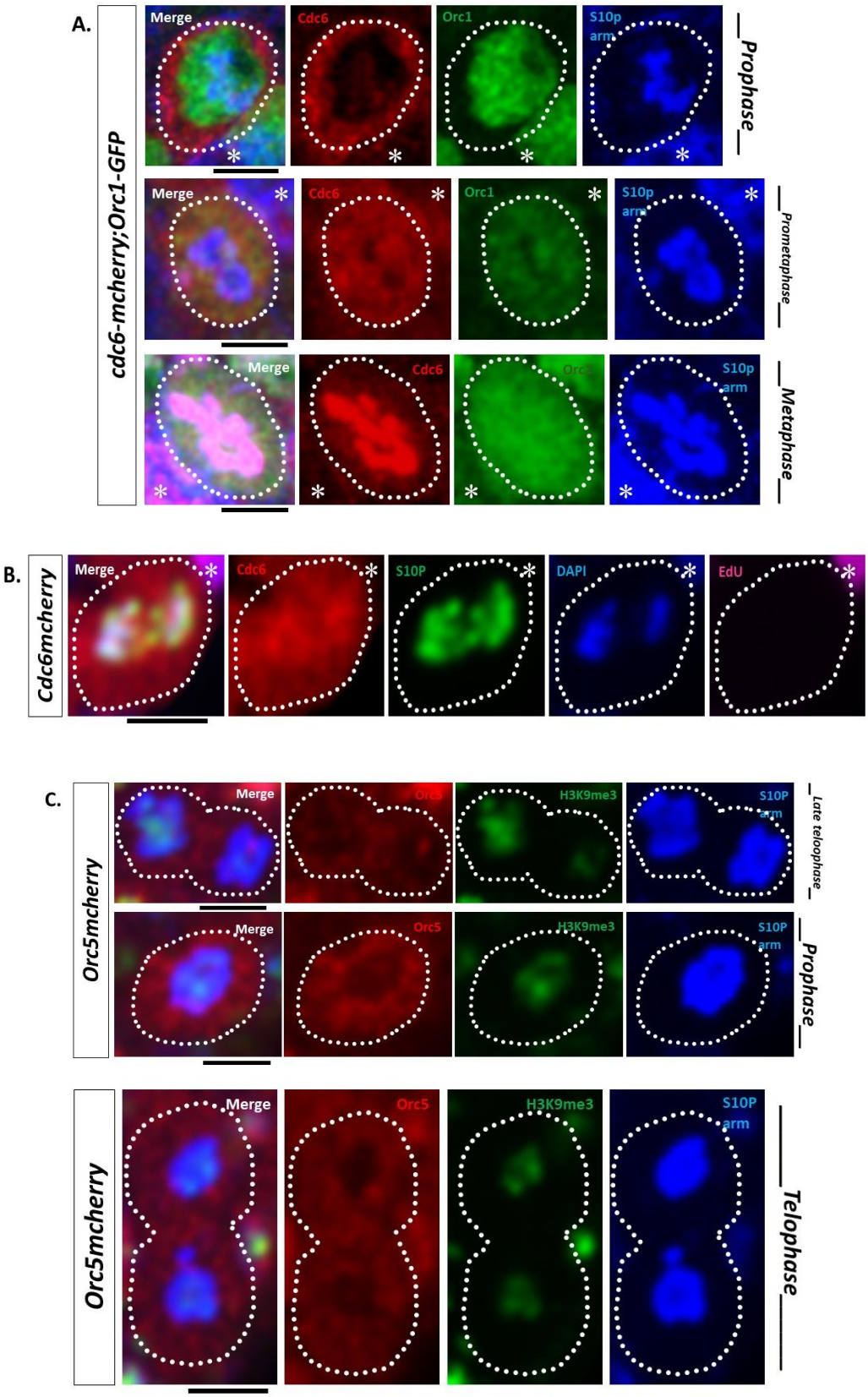

A.
cdc6-mcherry;Orc1-GFP
Merge
Cdc6
Orc1
S10p arm
Prophase
Prometaphase
Metaphase
B.
Cdc6mcherry
Merge
Cdc6
S10P
DAPI
EdU
C.
Orc5mcherry
Merge
Orc5
H3K9me3
S10P arm
Late telophase
Prophase
Telophase

Figure 9: Loading of DNA Replication Initiation Factors. (A) Cdc6-mcherry (red) and Orc1-GFP (green) binding through prophase, prometaphase, and metaphase. **(B)** Symmetric binding of Cdc6-mcherry (red) in anaphase between GSC and GB **(C)** Binding of Orc5-mcherry (red) in prophase, late telophase, and telophase. *=hub, scale bar is 5μm

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CAROLINA CHU

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Birth place: Maryville, Illinois USA

EDUCATION

Johns Hopkins University, Baltimore, MD *Expected May 2019*
Master of Science in Molecular and Cellular Biology

Johns Hopkins University, Baltimore, MD *Sept 2014-May 2018*
Bachelor of Science in Molecular and Cellular Biology

RESEARCH EXPERIENCE

Johns Hopkins University, Dr. Xin Chen *08/2017-present*
Research

regarding the mechanisms of the asymmetric DNA replication initiation between the stem and non-stem cells of the male germ cell line of *Drosophila* and the relation of this asymmetry to asymmetric histone inheritance

TEACHING EXPERIENCE

Johns Hopkins University, Cell Biology Lecture and Lab *01/2019-present*
Teaching Assistant

Johns Hopkins University, General Biology Lecture and Lab *08/2018-12/2018*
Teaching Assistant

VOLUNTEERING AND ORGANIZATIONS

- **My Sister's Place Women's Lodge**| Volunteer *01/2019-present*
- **Johns Hopkins Hospital** |Volunteer-Pediatric Research *01/2019-present*
- **Johns Hopkins Hospital** |Volunteer-PACU *10/2018-present*
- **Children's House** |Volunteer *10/2018-present*
- **Prometheus Journal** |Editor and Event Coordinator *10/2017-5/2018*
- **UAID** |Treasurer *09/2016-03/2018*

WORK EXPERIENCE

Johns Hopkins University, Department of Biology *02/2019-05/2019*
Student Note Taker-Graduate Virology

- take notes during Graduate Virology lecture and then upload my notes electronically to the Johns Hopkins Student Disability Service so that other students in the class who are unable to take notes will be able to have a record of what was said in lecture

Johns Hopkins Medical Institute, Wilmer Eye Institute *01/2016-06/2016*
Research Assistant

- cleaned and changed fish tanks for researchers' projects, picked out dead fish embryos too maintain health of others, created drug plates through drug dilutions and making different drug solution of varying concentrations, dispensed fish embryos in drug plates and observed the individual drug's effects on the ocular region of the embryo

Johns Hopkins Medical Institute, Department of Cell Biology *06/2015-09/2015*
Research Assistant

- changed *Drosophila* stocks for post-docs' projects, made apple juice caps for *Drosophila* embryo growth, aliquoted antibodies, prepared material for autoclaving process

Johns Hopkins Medical Institute, Department of Otolaryngology *01/2015-07/2015*
Lab Assistant

- helped post-docs with any tasks they asked such as washing glassware and making solutions, took care of paperwork, created equipment guidelines and research protocols, learned to operate JHU Core Store to order laboratory supplies, helped lab manager with administrative work

OTHER EXPERIENCE

Physician Shadowing

- **Dr. Alan Cohen, MD-Pediatric Neurosurgery, Johns Hopkins Hospital**
  - Occipital tumor resection 11/16/18 (4 hours)
  - Craniosynostosis 11/12/18 (5 hours)

- **Dr. Ernest Graham, MD-Obstetrics and Maternal/Fetal Medicine, Johns Hopkins Hospital**
  - Dilation and curettage, Vaginal Births, Emergency C-sections, Tubal Ligations, Induction, Epidural Placement 10/29-11/02/18 (20 hours)

- **Dr. Matthew DiGiustto, MD- Pediatric Emergency, Johns Hopkins Hospital**
  08/05/18 (5 hours)

- **Dr. Alejandro Garcia, MD-Pediatric Surgery, Johns Hopkins Hospital**
  - Pectus excavatum repair, hernia repairs 07/17/18 (6.5 hours)
  - Lung node removal, feeding tube insertion 07/09/18 (7.5 hours)

- **Dr. Kenton J. Zehr, MD-Cardiothoracic Surgery, Johns Hopkins Hospital**
  - Coronary artery bypass 06/29/2018 (8 hours)

AWARDS AND HONORS

- **Johns Hopkins University:** Dean's List Spring 2018
- **Johns Hopkins University:** Dean's List Fall 2018
- **Johns Hopkins University:** Dean's List Fall 2014

SKILLS

- **Techniques**: various laboratory procedures including the ones listed above along with procedures such as fluorescence imaging, animal dissections, PCR, gel electrophoresis…
- **Languages**: Native Chinese and English speaker
- **Programs**: proficient in imagej, Graphpad Prism, Microsoft Word, Excel, Powerpoint, Adobe Illustrator, and Adobe Photoshop